RESEARCH PLAN PROPOSAL

REPRODUCTIVE AND GENOTOXIC EFFECTS OF FLUORIDE, ALUMINIUM AND ITS COMBINATION IN MALE SWISS ALBINO MICE: AMELIORATIVE EFFECT OF *NIGELLA SATIVA* SEEDS

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INTRODUCTION

In order to provide a broad coverage of the mutagenic and presumably carcinogenic potential of metals, information is required on genotoxic effects at different levels, e.g. the gene, the chromosome and the cellular apparatus necessary for chromosome segregation. A number of testing procedures, *in vivo* have been designed to assess the effects of metals on the genetic material, consequently to assess the risk to living organisms including humans.

GENOTOXICITY

Genotoxicity is to determine the magnitude of genetic risk to man by an environmental agent/ chemical under a specified level of exposure. Assessment of the deleterious effects of chemicals or physical agents on the hereditary material and related genetic processes of living cells by altering the integrity and function of DNA at the gene or chromosomal level, the damage can lead to heritable mutations ultimately resulting in genetic disorders, congenital defects, or cancer. Targets of DNA damage include Somatic cells (detrimental to the exposed individual), germinal cells (potentially heritable effects) and mitochondria.

To assay for genotoxic molecules, researchers assay for DNA damage in cells exposed to the toxic substrates. This DNA damage can be in the form of single- and double-strand breaks, loss of excision repair, cross-linking, point mutations, and structural and numerical chromosomal aberrations (ATO, 2013).

REPRODUCTIVE TOXICITY

Reproductive toxicity is a hazard associated with some chemical substances which interfere in some way with normal reproduction, such substances are called reprotoxic. It includes adverse effects on sexual function and fertility in adult males and females, as well as developmental toxicity in offspring.

Reproductive toxicity is defined by the Globally Harmonized System as “adverse effects of chemicals on sexual function and fertility in adult males and females, as well as developmental toxicity in the offspring”.(Devi,2016). It causes alterations in sexual behavior, alterations to the reproductive organs and related endocrine system, reduced fertility, adverse pregnancy outcomes and modifications of other functions that are dependent on the integrity of the reproductive system.

FLUORIDE

Fluoride is the 13^{th} most abundant element on earth. It is an inorganic, monatomic anion of fluorine with the chemical formula F^- . It is the simplest anion of fluorine. Fluorine is never found by itself in nature but fluorides are found everywhere in soil, air, and water, as well as in plant and animal life. Its salts and minerals are important chemical reagents and industrial chemicals, mainly used in the production of hydrogen fluoride for fluorocarbons. In terms of charge and size, the fluoride ion resembles the hydroxide ion.

Fluoride, widely distributed in earth's crust is believed to constitute nearly 0.09% by weight of the upper lithosphere. Solid and gaseous fluorides are discharged into the atmosphere from the active volcanoes. They occur naturally in most waters and sometimes their natural concentration exceeds 20 mg/1. In U.S.A. alone about 350 thousand people are occupationally exposed to fluorides (IARC 1982). It is desirable in very limited quantities for healthy growth of bones and teeth preventing dental cavities, but in excess causes a disease known as fluorosis. Other deleterious effects of excess fluoride on human health are damage to kidney tubules due to continuous filtration of the ingested fluoride, dysfunction of reproductive system and ageing (Sharma *et al* 2007).

In India, fluorosis is endemic in 17 states due to excess fluoride content in the groundwater. Various anthropogenic sources contributing fluoride are steel, aluminum, ceramics and phosphate fertilizer production, as well as coal combustion (Smith and Hodge 1979; Hillier *et al.* 2000). Fluoride releasing compounds are also added for this purpose to a wide variety of dental products including toothpastes and mouthwashes. The public is exposed to fluoride ion by drinking fluoridated water and by using fluoride-containing dental products and treatments. Exposure may also occur through naturally present fluoride in foods and beverages, and in some cases by inhalation of fluoride compounds in the air (ATDSR, 1990).

Sources of fluoride include natural fluoride in foodstuffs and water, i.e., fluoridated water (usually at 1.0 mg/l), fluoride supplements (such as fluoride tablets), fluoride dentifrices (containing on average 1000 mg/kg), and professionally applied fluoride gel (containing on average 5000 mg/kg). The main source of fluoride for humans is the intake of groundwater contaminated by geological sources (maximum concentrations reaching 30–50 mg/l). The level of fluoride contamination is dependent on the nature of the rocks and the occurrence of fluoride-bearing minerals in groundwater. Fluoride concentrations in water are limited by fluorite solubility, so that in the absence of dissolved calcium, higher fluoride solubility should be expected in the groundwater of areas where fluoride-bearing minerals are common and vice versa. Endemic fluorosis is now known to be global in scope, occurring on all continents and affecting many millions of people (Susheela, 1999). In some regions, artificial fluorides used to fluoridate community water supplies (mostly at around 1 mg/l) include silicofluoride compounds (sodium silicofluoride and hydrofluosilicic acid) and sodium fluoride (NaF) (Chand, 2001).Even in the past, fluoride was considered an essential element. In actuality, there is a lack of consensus as to the role of fluoride in human nutrition optimal development and growth (Krishanmachari, 1986). It is now recognized that fluoride also affects cells from soft tissues, i.e., renal, endothelial, gonadal, and neurological cells (Jacobson and Montalbano 1985).

Effects of fluoride

Premature ageing is the overall effect of fluoride. The acceleration of ageing process occurred at the biochemical level by means of enzyme inhibition, collagen breakdown and disruption of immune system by inhibiting the migration rate of WBC cells to the infected area and genetic damages (Dunipace *et al* 1989). It also interferes with phagocytosis and induces release of super

oxide free radicals which damages the body leading to further acceleration of ageing process .The fluoride induces cancer and about 61,000 cancer death in USA resulted from fluoridation each year. Fluoride is more poisonous than lead and slightly less poisonous than arsenic. The rate of still born miscarriages by 4 months is extremely high in fluoride rich prone areas. It also causes nausea, bloody vomit, faintness, stomach cramps, tremors, skin rash, weight loss, constipation, aching bones etc. (Yiamouyiannis 1993).

USES OF FLUORIDE

- Preventing tooth decay - when fluoride is added to drinking water or included in toothpastes, mouthwashes, and other dental products.
- Treating osteoporosis (bone loss) - Fluoride taken by mouth continuously or cyclically (three months on, one month off) might increase bone mineral density, which is an indicator of bone strength.
- Fluoride seems to work better for improving bone strength in older women when combined with hormone replacement therapy. However, it's not clear whether taking fluoride actually reduces the chance of weak bones breaking.
- Preventing bone loss in people with rheumatoid arthritis.
- Preventing bone loss in people with Crohn's disease (an intestinal disorder).

ALUMINIUM

Aluminum is a chemical element in the boron group with symbol Al and atomic number 13. Aluminum (Al) is a trivalent cation found in its ionic form in most kinds of animal and plant tissues and in natural waters everywhere. It is a silvery-white, soft, nonmagnetic, ductile metal. Aluminum is the third most abundant element (after oxygen and silicon) in the Earth's crust, and the most abundant metal there. It makes up about 8% by mass of the crust, though it is less common in the mantle below. Aluminium metal is so chemically reactive that native specimens are rare and limited to extreme reducing environments. Instead, it is found combined in over 270 different minerals (Muller *et al* 1990).

Aluminium (Al) is the most widely distributed metal in the environment. Aluminum is the most prevalent metal and the third most important abundant element in earth's crust, only oxygen (49.5%) and silicon (26%) occur more commonly than aluminum (8%). In biological systems, Aluminum is present only in trace amounts (Piezent *et al* 2012). Al 3+ has been shown to have a definite biological function and it is poorly absorbed and efficiently eliminated when absorption does occur; Al is described mainly in bone, liver, testes, kidney, and brain. Al accumulation in tissues and organs results in their dysfunction and toxicity. Al is extensively used in daily life that provides easy exposure to human beings (Kumar and Gill 2009).

USES OF ALUMINIUM

Aluminium compounds are used in pharmaceuticals, in water treatment process, in utensils, in food additives and in consumer products. The sources of Al are especially corn, yellow cheese, salt, herbs, spices, tea, cosmetics, aluminium ware and containers. People are exposed to higher than normal levels of Aluminum through environmental pollution with the different aluminum containing compounds. Particulate matters distributed by cement producing factories contain high amount of Aluminum and populations residing in the vicinity are exposed to the pollution.

Aluminium is present in small amounts in mammalian tissues, yet it has no recognized physical role. On the contrary, its neurotoxic effect on living organisms is becoming clear, aluminium being implicated as interfering with a variety of cellular metabolic processes in the nervous system and in other systems. Although molecular mechanisms by which aluminium exerts its neurotoxicity remain to be established, several pieces of evidence suggest that Aluminium can interfere with cellular metabolism in terms of biological stimulation, inhibition, or metal accumulation and compartmentation (Zattal *et al* 1991).

Aluminium is ubiquitous in the environment and its extensive industrial utilization has stimulated considerable interest in the possible environmental toxicity of this metal. When Al is mobilized from soil by acid rain, it poses a hazard to all exposed organisms (Beavon 2004, Birchall *et al* 1989). Aluminium compound have been used for 30 years to control phosphate levels in patients undergoing haemodialysis. The toxic effect arising from the absorption and accumulation of aluminium have well been documented and include a progressive encephalopathy which eventually leads to dementia. The hippocampus is a major component of the brains of human and other mammals. It belongs to the limbic system and plays important roles in long-term memory and spatial navigation (Bradbury, 1992).

EFFECTS OF COMBINATION OF FLUORIDE AND ALUMINIUM

Aluminium and fluoride are both known to be potential environmental hazards. They are found together in the earth's crust in the form of cryolite (Na_3AlF_6).

Studies on the combined effects of aluminium (Al) and fluoride (F) in biological systems are very controversial. Aluminium and fluoride are mutually antagonistic in competing for absorption in the gut. Aluminium is known to decrease the intestinal absorption of fluoride and to increase its excretion in humans, thereby reducing its toxicity (ATDSR, 1997). However, fluoride enhances digestive absorption of aluminum (Allain *et al* 1996). The combined effects of aluminium and fluoride aggravated toxicity in the blood and femur bone of male chicks (Dai *et al* 1994). Changes in the behavioral pattern of rats following chronic aluminium fluoride (AlF_3) administration are known (Varner *et al* 1994). The aluminium levels in brain and kidney of rats were found to be high and the extent of alterations in neuronal density as well as

cerebrovasculature were greater in animals exposed to AlF_3 than to NaF and greater in the NaF group than in controls (Jensen *et al* 1998). Fluoride and Aluminium combined toxicosis is endemic in the severe fluorosis regions of the Shuicheng area of Guizhou province in South China (Wei *et al* 1994).

In a differing view, consumption of drinking water containing elevated but non-toxic levels of fluoride has been proposed for the prevention of Alzheimer's disease (AD) as aluminum is one of the contributing factors in the occurrence of this disease (Kraus and Forbes 1992). Fluoride causes neurotoxicity in rats, and the effects were greater in early stage of intoxication. (Chlubek *et al* 1998, Mullenix *et al* 1995) In a study on male and female rabbits, the lipid metabolism in the brain was altered due to fluoride intoxication, and the neurons of the cerebellar cortex showed degeneration (Shashi *et al* 1994).

The present study plans to evaluate the genotoxic effects of combination of fluoride and aluminium, in somatic cells and germ cells of male mice. The study assumes importance in view of widespread human exposure to both metals in combination.

NIGELLA SATIVA SEEDS

Nigella sativa (black-caraway, also known as nigella or kalonji), often called black cumin.(Heiss and Andreas, 2005) is an annual flowering plant in the family Ranunculaceae, native to south and southwest Asia. *Nigella sativa* grows to 20–30 cm (7.9–11.8 in) tall, with finely divided, linear (but not thread-like) leaves. The flowers are delicate, and usually colored pale blue and white, with five to ten petals. The black caraway fruit is a large and inflated capsule composed of three to seven united follicles, each containing numerous seeds which are used as spice, sometimes as a replacement for black cumin (Bunium bulbocastanum). Millions of people in the Mediterranean region and on the Indian subcontinent use the oil from the seed of *N. sativa* daily as a natural protective and curative remedy. The seeds are very rich and diverse in chemical composition. They contain amino acids, proteins, carbohydrates, fixed and volatile oils (Khan, 1999). It is mainly composed of thymoquinone (30–48%), which has many therapeutic effects including antioxidant properties (Salem, 2005). It has the ability to significantly overcome illness and maintain health if taken regularly, and it acts on the principle of assisting the body's own natural healing process (Mayali, 2007) *N. sativa* has been used for thousands of years as a spice and food preservative. The oil and seed constituents, in particular thymoquinone, have shown potential medicinal properties in traditional medicine (Omar, 1999).

Nigella sativa seeds have shown several therapeutic effects such as prevention of cancer, antihypertensive effect, anti-inflammatory, analgesic and antihistaminic actions (Swamy and Tan, 2000; Tahir *et al*, 1993; Abdel and Fattah, 2000; Chakravarty, 1993 & Khan, 1999). *Nigella sativa* seed composition includes nutritional components such as carbohydrates (glucose, xylose, rhamnose, andarabinose), vitamins as thiamine, riboflavin, pyridoxine, niacin and folic acid, mineral elements (calcium, iron and potassium), proteins, alkaloids (nigellidine, nigellimine and nigellicine), 36%-38% fixed oil and

0.4%-2.5% essential oil (Hosseinzadeh and Parvardeh, 2004). *Nigella sativa* possesses main components known as thymol and quinones (thymoquinone, thymohydroquinone, dithymoquinone (Randhawa *et al* 2011).

REVIEW OF LITERATURE

Genotoxicity

Vijayalaxmi and D'Souza (2004) investigated the genotoxicity of platinum antitumor agent carboplatin (Cbp) in mouse. Cyclophosphamide (50 and 100mg/kg b.w) was used as positive control and distilled water formed the negative control. Carboplatin induced significant MN both in the fetal liver and the maternal bone marrow at different doses. It also induced high frequency of abnormal sperms at higher doses and the effect was significant at all the doses tested.

Cavusoglu *et al* (2010) investigated the frequency of MN in erythrocytes and body weight gain in 80 *Mus musculus* var. *albinos* exposed to 10Gy gamma (γ) radiation and heavy metal ions. MN assay was used as an indicator of genotoxicity induced by γ–radiation and heavy metal toxication. As a result, the frequency of MN was higher in the Hg, Pb and γ–radiation treated animals than animals in control group. Besides, MN frequency was higher in mice exposed to γ–radiation than in Hg and Pb treated mice, and differences was statistically significant ($p<0.005$). Histopathologically, periaciner necrosis, hydropic degenerations in the liver and villous atrophy in the intestine, gastric glandular mucosae necrosis were observed in treatment groups. These results indicate that MN assay is very sensitive and a useful biomarker for the evaluation of the genotoxicity

Hanan *et al* (2012) investigated the most toxic effects of lead with trial to diminish this toxicity by supplementation of casein or activated charcoal. The significant increase in serum parameters and decreased total protein, HLDL, VLDL, trace mineral salts, SOD and GSH- px were observed. The results of haematological study evoked a significant decrease in the red blood cells (RBCs) count, blood haemoglobin (Hb) concentration and packed cell volume (PCV) and observed significant increase in the percent of multinucleated polychromatic erythrocytes (MPCE) as well as polychromatic- erythrocytes (PCE) and normochromatic erythrocyte (NCE) ratio (PCER/NCE) in cytogenetic study. Lead concentration in level serum and different tissues showed lead accumulation in serum, kidney, liver, muscle, intestine and spleen and also observed charcoal and casein induce protective effects against lead toxicity in most of above parameters.

Mathew and Thoppil (2012) observed the biochemical and genotoxic effects of Arsenic and studied its distribution in selected tissues of Sprague-Dawley rats. Arsenic exposure significantly increased the activities of serum parameters and the number of structural chromosomal

aberrations (SCA) and frequency of micronuclei (MN) in the bone marrow cells. In contrast, the mitotic index in these cells was significantly reduced ($p<0.05$). Our results also demonstrate that Arsenic has a strong genotoxic potential.

Makawy *et al* (2015) evaluated the protective role of mango peel or kernel defatted extracts against Lead acetate adverse effects on oxidant/antioxidant status, liver dysfunction biomarkers, histopathological changes and genotoxicity in male mice Mango extracts prevented the histopathological changes in liver induced by Lead acetate and decreased the cytotoxicity of lead by increasing the ratio of PCE/NCE. Mango extract treatment reduced the DNA damage induced by Lead acetate in liver as demonstrated by a reduction in micronuclei and decrease in tail length, tail DNA% and Olive tail moment

Reproductive Toxicity

Sekhar *et al* 2011 studied the synergistic effect of sodium fluoride and cypermethrin on the somatic index and histopathology of albino mice testes. Separate or combined treatment resulted in a significant ($p<0.05$) decrease in body, testis, and epididymis weights, along with histopathological changes in the testis, especially after 30 days. The changes were greater in combination than separately, possibly because of a synergistic effect of cypermethrin and sodium fluoride.

Madhavi *et al* (2007) evaluated the protective effect of *Phyllanthus emblica* against clastogenicity induced by lead nitrate on the incidence of sperm head abnormalities in the germ cells of mice. At higher concentration of lead, a significant increase in the percentage of sperm head abnormalities was noted but when animals primed with Phyllanthus fruit extract (PFE), a reduction in the frequency of sperm head abnormalities was observed.

Sujayraj *et al* (2016) evaluated toxic potency of cyclophosphoamide in male reproductive system comparing between acute and sub-chronic therapeutic doses. The semen samples were analyzed for sperm abnormality, count and motility, by following the standard protocols. Gonado-somatic index, i.e., ratio of the body weight to testes weight was also determined. Both the acute and subchronic doses of CP induced the adverse effects on reproductive system in terms of high frequency of abnormal sperm, reduction in sperm count and motility at the significant levels when compared with the solvent control ($p<0.001$). Comparison of the results between acute and subchronic treated groups indicated that the magnitude of the CP-induced effects on sperm morphology, count and GSI ($p<0.05$) was statistically found to be higher in the latter group. Further, the effect of subchronic dosing was statistically found to be more on the sperm motility ($p<0.01$). Thus, subchronic dosing of CP even at a lower dose imparts more toxic effects on the testes and spermatogenesis than that of the acute higher dose treatment.

Yılmaz and Erkan (2015) demonstrated the oxidative stress and apoptosis in Sertoli cells that had been exposed to sodium fluoride (NaF). Cell viability, cell proliferation, cytotoxicity, lipid peroxidation, and reactive oxygen species were measured and the apoptosis/necrosis rate was calculated. A significant decrease in the Sertoli cell viability was found with the high dose of NaF (20 ppm). The fluoride ion also caused oxidative damage by increasing cytotoxicity, reactive oxygen species, and lipid peroxidation in a dose- and time-dependent manner. The apoptosis/necrosis rate was significantly increased in both NaF groups. Vitamin C, a strong antioxidant, had a protective effect against the fluoride-induced damage in the Sertoli cells including the increased apoptosis/necrosis rate. The study showed that oxidative stress plays a possible role in the apoptosis of Sertoli cells induced by NaF, and that these effects can be suppressed with vitamin C.

Chinoy *et al* (2005) observed the genotoxicity studies of Aluminium chloride and Sodium fluoride independently and concurrently, was carried out on human peripheral blood lymphocyte *in vitro*. The study showed that Sodium fluoride and Aluminium chloride independently were significantly toxic in the *in vitro* system. The combined treatment was more profoundly toxic when compared to individual toxicity of the chemical on various parameters studied, without metabolic activation. The results suggest that NaF and AlCl3 have a genotoxic and cytotoxic potential in human peripheral blood cultures *in vitro*.

Sharma N. and Bhattacharya L. (2014) investigated the possible involvement of oxidative stress in the suppression of steroidogenesis. Testicular steroidogenic activity was evaluated by measuring the activities of two steroidogenic key enzymes, 65–3β hydroxysteroid dehydrogenase (3β–HSD), 17β–hydroxysteroid dehydrogenase (17β–HSD) and serum levels of testosterone and cholesterol in albino mice. Administration of lead acetate at a dose of 1.25mg/kg body weight for 45 days lowered the weights of testes, decreased testicular 65–3β – HSD and 17β– HSD activities and serum levels of cholesterol and testosterone. Coadministration of vitamin E (2 mg/kg BW) to the lead treated group restored all the parameters cited above to near the control values. Therefore, this study revealed that vitamin E has beneficial effects against lead induced steroidogenesis testicular toxicity.

Khan *et al* 2013 studied the ameliorative potential of curcumin (CMN) against arsenic (As) induced reproductive effects in male rats. Administration of Arsenic (40 ppm in drinking water for 28 days) caused increase in lipid peroxidation and reduction in glutathione and activities of catalase, superoxide dismutase and histopathological impairments in testes. As caused decrease in reproductive organs weight, sperm count, motility, testosterone level and increase in sperm abnormalities, sperm DNA damage with negative fertility index. As induce apoptosis by ROS generation, loss of mitochondrial transmembrane potential, upregulation of Bax, caspase-9 and -3 and repression of Bcl2 mRNA level in mitochondrial pathways. As lowered 17β-hydroxysteroid dehydrogenase activity and caused down regulation of steroidogenic acute

regulatory protein and Cytochrome P450 enzyme mRNA level. Curcumin co-treatment (100 mg/kg body weight orally for 28 days) was found to be effective in reversing arsenic-induced all these effects and could be able to antagonize reproductive toxicity.

Madhavi *et al* 2007 evaluated the protective effect of *Phyllanthus emblica* against clastogenicity induced by lead nitrate on the incidence of sperm head abnormalities in the germ cells of mice. At higher concentration of lead, a significant increase in the percentage of sperm head abnormalities was noted but when animals primed with Phyllanthus fruit extract (PFE), a reduction in the frequency of sperm head abnormalities was observed.

Reproductive and genotoxicity

Çelikler *et al* (2007) aimed to compare mutagenic potential of pyrimethamine in mammalian spermatogonial and bone marrow cells. Pyrimethamine was found to produce a significant increase in structural chromosomal aberrations after acute treatment in bone marrow cells of mice (p 0.001). It also induced chromosome abnormalities in spermatogonial cells (p 0.05) at the highest dose.

Mohapatra *et al* (2013) Oral administration of cadmium chloride caused DNA damage in the tissues of testes, liver and kidneys. Marked changes in the histological architecture of these organs were observed. Distortion of seminiferous tubules, desquamation of spermatogonia, spermatocytes and spermatids with cytoplasmic vacuolization and nuclear fragmentation in testes; disruption in arrangement of hepatic plates, widespread fibrosis, interstitial mononuclear pycnotic nuclei in the liver and severe multifocal cloudy, hydropic degeneration with loss of cellular integrity in the renal tissues were observed. It was observed that the severity of pathological effects was dependent on the duration of post treatment period.

Protection by *Nigella Sativa Seeds*

Rahman *et al* (2013) evaluated the effect of ethanol extract of *Nigella sativa on* cyclophosphamide reproductive toxicity and acrosome function of sperm. Effects of cyclophosphamide include reduction of weights of testes, epididymides and sperm count. A higher value of sperm count was observed in the cyclophosphamide plus *Nigella sativa* group when compared to the cyclophosphamide alone group, perhaps indicating the medicinal plant antioxidant properties in the germ cell line. Treatment with cyclophosphamide led to a high percentage of acrosome reacted sperm possibly to spontaneous acrosomal reaction caused by free radicals. Although the combination treatment seemed to show similar histology to the effects of cyclophosphamide alone, a protective outcome was indicated with the higher number of sperm count with the addition of *Nigella sativa*. Paradoxical effects of *Nigella sativa* were observed on

the cells in the testis and acrosome reaction including in the protection against oxidative agents and alkylating effects of cyclophosphamide.

Parveen and Shadab (2011) tested *in vivo* effect of *Nigella sativa* (50 mg/kg b.wt.) against the genotoxicity of FeSO4.7H2O. Bone marrow cells were obtained from the rats. The results of treatments with *Nigella sativa* indicated that it statistically significantly decreases the number of chromosomal aberrations with aberrations induced by FeSO4.7H2O, but it cannot completely protect cells from damage. *Nigella sativa* showed the most efficient anticlastogenic effect during simultaneous treatment with FeSO4.7H2O. The result further strengthened the findings about the protective and antigenotoxic properties of *Nigella sativa.*

Hussein *et al* 2014 aimed to investigated the possible role of Nigella Sativa Oil in ameliorating the toxic effect of Aluminium Chloride on the histological structure of the testis in male rats. Aluminum chloride ($AlCl_3$) is an environmental xenobiotic that induces free radical-mediated cytotoxic and reproductive toxicity. *Nigella sativa* oil (NSO) is an antioxidative agent reported to be important for detoxification. Compared with the control group, administration of $AlCl_3$ revealed significant changes in the seminiferous tubules, which appeared shrunken with disorganized germinal epithelium. Cellular vacuolations as well as sloughed spermatogenic cells into the lumen were observed. The interstitium was widened with interstitial hyperplasia. Ultrastructural examination of the $AlCl_3$ exposed group revealed cells with dense cytoplasm and heterochromatic nuclei. Late differentiating spermatids showed deformed heads with widening of the subacrosomal space and redundant acrosome. Coadministration of NSO with $AlCl_3$ showed amelioration of most of the histological changes in the $AlCl_3$-intoxicated group. The withdrawal group showed persistence of many structural changes noticed in the $AlCl_3$-intoxicated group with a significant difference compared with the $AlCl_3$+NSO-treated group.

Nailey (2010) investigated some histopathological changes produced by cimetidine on male reproductive organs in mice and extended to examine the aqueous suspension of powdered *Nigella sativa* seeds have ability to reduce this toxicity. Animals which were treated with cimetidine only along the period of experiment showed sever pathological changes, particularly in testes and epididymis, whereas oral administration of the aqueous suspension of Nigella seeds aqueous suspension significantly reduced these changes which reflected by obvious increment of spermatogonia and reducing the damage of seminiferous tubules. The protective activity of *Nigella sativa* has been resumed via diminishing the reproductive histopathological changes associated with long-term of treatment with cimetidine.

OBJECTIVES:

- To evaluate the Genotoxic potential of fluoride, aluminium and its combination in Swiss albino mice.
- To assess the reproductive toxicity caused by combination of metals and its amelioration by *Nigella Sativa* seeds.

MATERIALS AND METHODS:

Experimental Animal: Swiss albino mice

Rodents are small, easily housed and maintained and adapt well to new surroundings. They also reproduce quickly and have a short lifespan of two to three years, so several generations of mice can be observed in a relatively short period of time.

Most of the mice used in medical trials are inbred so that other than sex differences, they are almost identical genetically. This helps make the results of medical trials more uniform, Aaccording to the National Human Genome Research Institute. As a minimum requirement mice used in experiments must be of the same purebred species.

Swiss Albino Mice 4-6 weeks old will be procured from the animal house approved by the Ethical Committee CPCSEA Registration number No: 1689/PO/9/13/CPCSEA. The animals will be maintained on standard diet and water *ad libitum* at the Animal House Laboratory and housed in a temperature- controlled 25± 3.2ºC on light/dark cycle of 12/12 hours and artificially illuminated room, free from any source of chemical contamination.

Drug: Fluoride, Aluminium and Combination of Aluminium and Fluoride in different concentrations will be given orally to the mice.

Protective Agent: *Nigella Sativa* seeds

There will be three sets of experiment

1) Sodium Fluoride (NaF) treated:

Group	Treatment and dose	Duration of treatment (days)	Day of autopsy	No of animals used in each group
I	Control (Vehicle i.e. Distilled water)	30	Along with the treated groups	10
II	Sodium fluoride (NaF)	15, 30	16^{th} , 31^{st}	10
III	NaF as in Group II for 30 days + protective (dose 1) for next 30 days	45, 60,	46^{th} , 61^{st}	10
IV	NaF as in Group II for 30 days + protective (dose 2) for next 30 days	45, 60	46^{th} , 61^{st}	10
V	Simultaneously administration of NaF + Protective (dose1) for 15, 30,45, 60 days	15, 30,45, 60	16^{th} , 31^{st}, 46^{th} , 61^{st}	10
VI	Simultaneously administration of NaF + Protective (dose2) for 15, 30,45, 60 days	15, 30,45, 60	16^{th} , 31^{st}, 46^{th} , 61^{st}	10

2) Aluminium Chloride ($AlCl_3$) treated:

Group	Treatment and dose	Duration (days)	Day of autopsy	No of animals used
I	Control (Vehicle i.e. Distilled water)	30	Along with the treated groups	10
II	Aluminium Chloride ($AlCl_3$)	15, 30	31^{st}	10
III	$AlCl_3$ as in Group II for 30 days+ protective	45, 60	46^{th} , 61^{st}	10

	(dose 1) for next 30days			
IV	$AlCl_3$ as in Group II for 30 days + protective (dose 2) for next 30 days	45, 60	46^{th} , 61^{st}	10
V	Simultaneously administration of $AlCl_3$+ Protective (dose1) for 15, 30,45, 60 days	15, 30,45, 60	16^{th} , 31^{st}, 46^{th} , 61^{st}	10
VI	Simultaneously administration of $AlCl_3$+ Protective (dose2) for 15, 30,45, 60 days	15, 30,45, 60	16^{th} , 31^{st}, 46^{th} , 61^{st}	10

3) Sodium Fluoride + Aluminium Chloride ($NaF+AlCl_3$) treated

Group	**Treatment and dose**	**Duration (days)**	**Day of autopsy**	**No of animals used**
I	Control (Vehicle i.e. Distilled water)	30	Along with the treated groups	10
II	Sodium fluoride ($NaF+ AlCl_3$)	30	31^{st}	10
III	$NaF+AlCl_3$ as in Group II for 30 days + protective (dose 1) for next 30 days	45, 60	$46^{th,}$ 61^{st}	10
IV	$NaF+AlCl_3$ as in Group II for 30 days + protective (dose 2) for next30 days	45, 60	$46^{th,}$ 61^{st}	10
V	Simultaneously administration of $NaF+ AlCl_3$ +Protective (dose 1) for 15, 30,45, 60 days	15, 30,45, 60	16^{th} , 31^{st}, $46^{th,}$ 61^{st}	10
VI	Simultaneously administration of $NaF+ AlCl_3$ +Protective (dose 2) for 15, 30,45, 60 days	15, 30,45, 60	16^{th} , 31^{st}, $46^{th,}$ 61^{st}	10

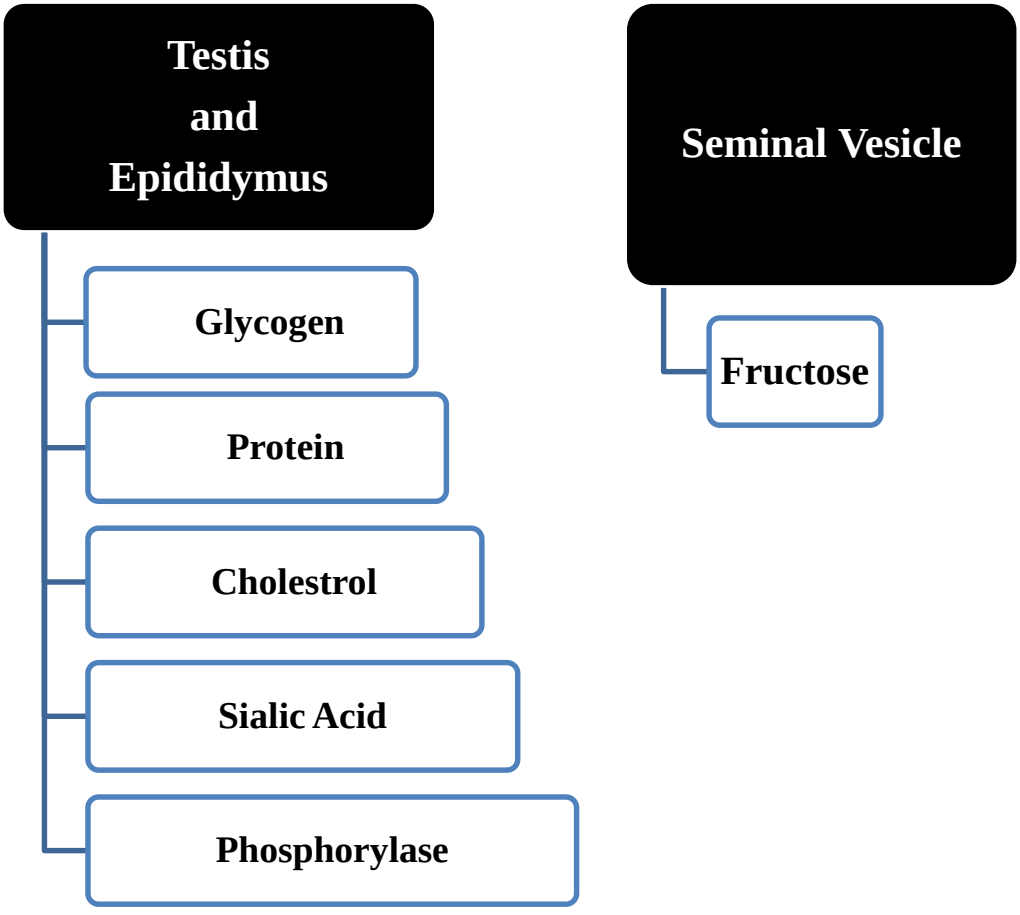
SAMPLING SCHEDULE

After the above treatments of the last dose for each group, mice will be tested for genetic and histopathological analyses. Animals will be injected intraperitoneally with 0.5 ml of colchicine (0.05 %) solution for two hours before sacrifice. Then, animals will be sacrificed by cervical dislocation. Immediately, bone marrow, testis, epididymus and Seminal vesicle will be collected.

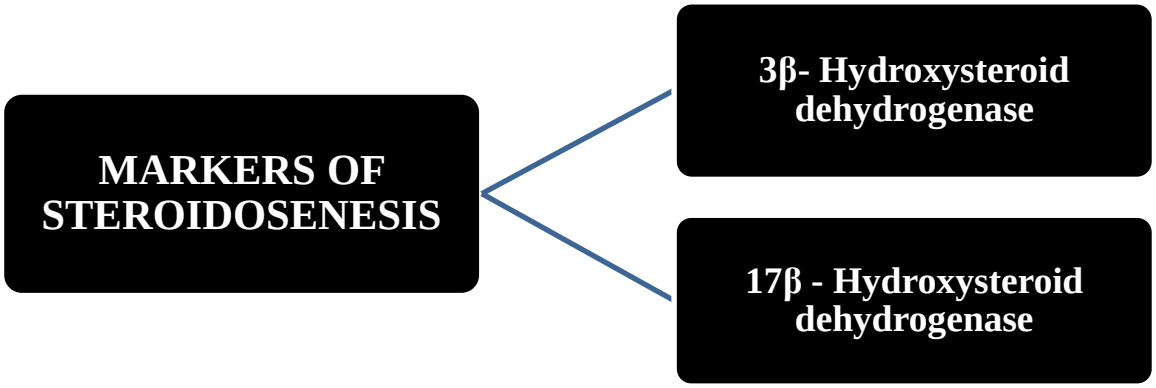
Bone marrow samples will be used to study the chromosome aberrations and mitotic index. Testis cells will be examined for sperm morphology analysis and histopathological changes. Epididymus and testis will be examined for biochemical parameters.

PARAMETERS:

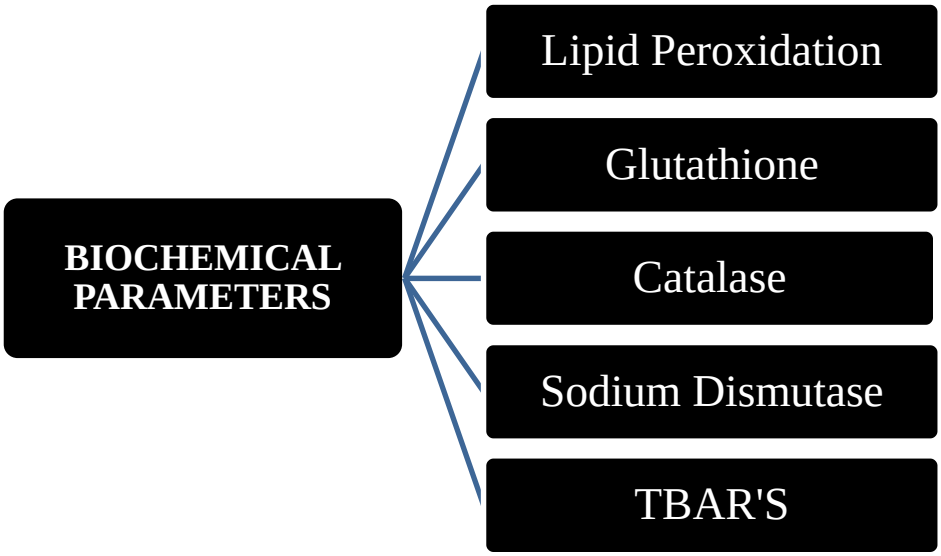
1) Biochemical parameters: Testis and Epididymus



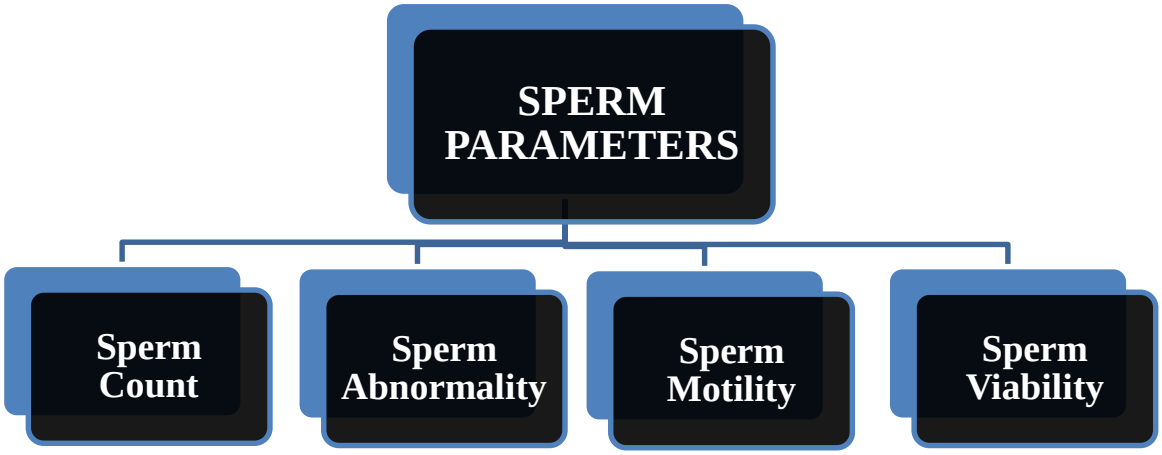
2) Markers of Steroidogenesis



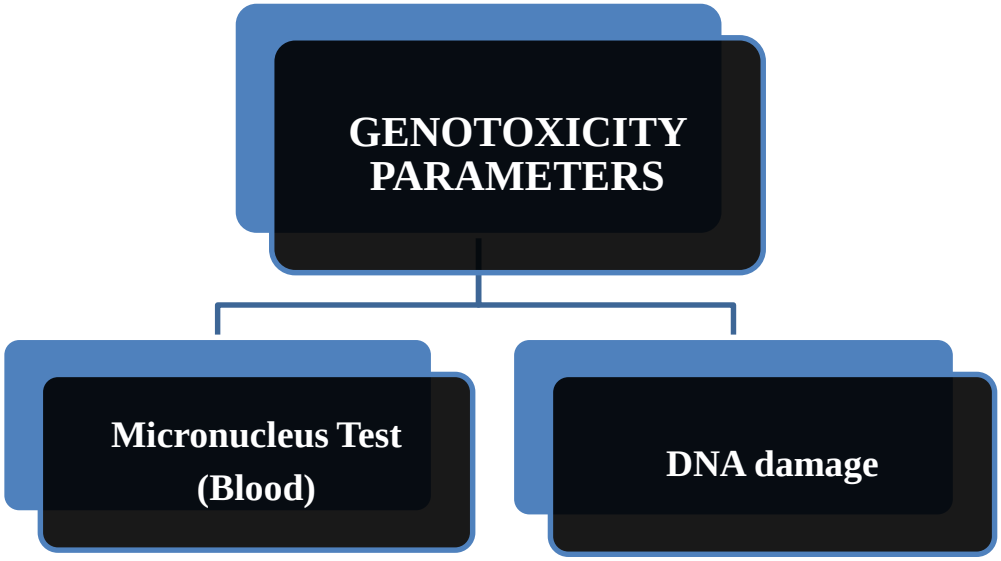
3) Biochemical Parameters



4) Sperm parameters



5) Genotoxicity parameters



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BIOCHEMICAL PARAMETERS: TESTIS AND EPIDIDYMUS

Protein
Protein levels in testis and epididymides of control and all treated groups of animals will be estimated by the method of Lowry *et al* (1951).

Glycogen
Glycogen levels in vas deferens of control and all treated groups of mice will be estimated using the method of Seifter *et al* 1950.

Sialic Acid
A periodate resorcinol method Jourdian *et al*, 1971 will be used for the quantitative determination of free and glycosidically bound sialic acids.

Phosphorylase
The activity of the enzyme phosphorylase in vas deferens of control and all treated groups of mice will be assayed by the method of Cori *et al* 1943.

Cholestrol
Estimation of cholesterol was done by the method of Zlatki *et al* (1953) using Ferric chloride as the colouring reagent

MARKERS OF STEROIDOGENESIS

3β- Hydroxysteroid Dehydrogenase (3β-HSD)
The testicular 3P-hydroxysteroid dehydrogenase in control and all treated groups of mice was assayed by the method of Talalay (1962).

17 -β Hydroxysteroid Dehydrogenase (17-β hsd)
The testicular 17p-Hydroxysteroid dehydrogenase activity in control and all treated groups of mice was assayed by the method of Talalay (1962). Testicular steroidogenic activity is measured by the activities of two steroidogenic key enzymes,3β hydroxysteroid dehydrogenase (3β–HSD) and 17β–hydroxysteroid dehydrogenase (17β–HSD).

Fructose
Fructose concentration in the seminal vesicles was estimated by the method of Foreman *et a1* 1973.

SPERM PARAMETERS:

Sperm abnormality assay: The Sperm abnormality assay will be assessed for morphological abnormalities by Bruce *et al* 1974.

Sperm Count and Sperm Motility: Sperm count in cauda epididymis of control and all treated groups of mice will be determined using the Neubauer Chamber of haemocytometer according to the method of Prasad *et al* 1972.

Sperm Viability: The percentage of the live spermatozoa will be determined using 1% trypan blue (supravital stain) as described by the method of Talbot and Chacon, 1981.

HORMONAL ASSAY:

Testosterone: The blood samples were obtained from the animals by heart puncture. The serum level of testosterone will be determined by ELISA KIT.

Luteinizing hormone: The blood samples were obtained from the animals by heart puncture. The serum level of testosterone will be determined by ELISA KIT.

GENOTOXICITY PARAMETERS:

Micronucleus Assay in Blood: The micronucleus assay in blood will be conducted according to Chauhan *et al* 2000.

HISTOPATHOLOGICAL EXAMINATION:

Testis will be dissected from all animals immediately after killing, washed thoroughly with formal saline and then fixed in 10% neutral-buffered formal saline for 72 hours at least and then will be processed for histopathological slides.

STATISTICAL ANALYSIS:

Statistical analysis was performed with SPSS software. Data will be analyzed using one-way analysis of variance (ANOVA) followed by t-test for comparison between different treatments in same sex. The values were expressed as mean ± S.E. and difference were considered as significant when $P< 0.05$.

SIGNIFICANCE OF THE STUDY

The study will help in identifying the genotoxic and reprotoxic nature of combination of metals and its implications further in curing and nullifying these effects with the *Nigella Sativa* seeds.

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